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Vaccinium angustifolium Root Extract Suppresses FcεRI Expression in Human Basophilic KU812F Cells.

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ABSTRACT: *Vaccinium angustifolium*, commonly known as the lowbush blueberry, is a rich source of flavonoids, with which various human physiological activities have been associated. The present study focuses on the investigation of the effect of the methanolic extract of *V. angustifolium* root extract (VAE) on high affinity immunoglobulin E receptor (FcεRI) α chain antibody (CRA-1)-induced allergic reaction in human basophilic KU812F cells. The total phenolic content of VAE was found to be 170±1.9 mg gallic acid equivalents/g. Flow cytometry analysis revealed that the cell surface expression of FcεRI was suppressed in a concentration-dependent manner upon culture with VAE. Reverse-transcriptase polymerase chain reaction analysis showed that the mRNA level of the FcεRI α chain was reduced in a concentration-dependent manner as a result of VAE treatment. Western blot analysis revealed that the protein expression of FcεRI and the phosphorylation of extracellular signal-regulated kinases (ERK) 1/2 were concentration-dependently inhibited by VAE. We determined that VAE inhibited anti-CRA-1-induced histamine release, in addition to the elevation of intracellular calcium concentration ($[Ca^{2+}]i$), in a concentration-dependent manner. These results indicate that VAE may exert an anti-allergic effect via the inhibition of calcium influx and histamine release, which occurs as a result of the down-regulation of FcεRI expression through inhibition of ERK 1/2 activation.

Keywords: *Vaccinium angustifolium*, FcεRI, ERK 1/2, calcium influx, histamine

INTRODUCTION

Blueberry is a flowering plant of the *Vaccinium* genus, and many species of blueberry are rich sources of anthocyanins and other flavonoids, which have been shown to exert a variety of beneficial effects in the protection against inflammation, carcinogenesis, and chronic diseases (1-8). Among the *Vaccinium* genus, the lowbush blueberry, *Vaccinium angustifolium*, is native to Eastern and Central Canada, and the North East of the United States. This species has been used for the treatment of diabetic symptoms, and it is evidenced to possess a variety of physiological properties including anti-inflammatory, antioxidant, and anticancer activities (9-16). We previously reported that *V. angustifolium* root extract (VAE) inhibited A23187 and phorbol 12-myristate 13-acetate-induced degranulation via down-regulation of protein kinases C translocation (17). The regulation of expression of FcεRI, a high affinity immunoglobulin E receptor, by VAE has not been investigated.

FcεRI is expressed on the cell surface of mast cells and basophils, and it performs a crucial function in IgE-mediated allergic responses (18,19). The aggregation of FcεRI by multivalent allergen (Ag)-IgE antibody (Ab) complexes, or by an anti-FcεRI-Ab, is the major stimulus for the initiation of the activation signal cascade, which triggers degranulation and results in the release of inflammatory mediators including histamine, in turn inducing an allergic response such as asthma, atopic dermatitis, and allergic rhinitis (20,21). The FcεRI molecule expressed on mast cells and basophils is a tetrameric receptor composed of one α, one β, and two disulfide-linked γ chains. Among these three subunits, the α chain of FcεRI is a specific component that largely extends out to the extracellular region and binds directly and with high affinity to the Fc portion of the IgE antibody (22). Thus, the suppression of FcεRI expression on the surface of mast cells and basophils may result in an attenuation of the IgE-mediated allergic response. In the present study, we assessed the suppressive effects of VAE on FcεRI expression in human basophilic KU812F cells.

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MATERIALS AND METHODS

Chemicals

Roswell Park Memorial Institute (RPMI)-1640 and heat-inactivated fetal bovine serum (FBS) were purchased from HyClone Laboratories (Logan, UT, USA). The anti-FcεRI α chain antibody (CRA-1) was purchased from Kyokuto (Tokyo, Japan). Mouse IgG antibody was purchased from Biosources (Burlingame, CA, USA). Anti-mouse IgG fluorescent isothiocyanate (FITC) antibody was purchased from Jackson ImmunoResearch Laboratories, Inc. (Baltimore, PO, USA). Antibiotics and antimycotics were purchased from Gibco BRL (Gaithersburg, MD, USA). TRI_{ZOL} reagent was purchased from Invitrogen (Carlsbad, CA, USA). Oligo $(dT)_{15}$ primer, moloney murine leukemia virus (MMLV) reverse transcriptase, GoTaq DNA polymerase, and CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay were obtained from Promega (Madison, WI, USA). Protease inhibitor cocktail was purchased from Roche Diagnostics GmbH (Penzberg, Germany). β-Actin, anti-phosphorylated extracellular signal-regulated kinases (ERK) 1/2 and ERK 1/2 antibodies, and the horseradish peroxidase (HRP)-conjugated secondary antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Chemiluminescence detection reagents were purchased from Perkin Elmer (Waltham, MA, USA), and the polyvinylidene difluoride (PVDF) membrane was purchased from Millipore (Bedford, MA, USA). All other reagents, including hydroxyethyl piperazinylethanesulfonic acid (HEPES), L-glutamine, kaempferol, fura 2-acetoxymethyl (AM), histamine, and *o*-phthalaldehyde (OPA) were purchased from Sigma Chemicals (St. Louis, MO, USA).

Preparation of extract

The roots of *V. angustifolium* were obtained from Quebec, Canada, and the dried and powdered samples were mixed with 10 volumes of methanol for extraction. The extract was then centrifuged, filtered, concentrated under a vacuum, and lyophilized. The lyophilized extract was stored at −20°C and dissolved in dimethyl sulfoxide prior to use.

Total phenolic content (TPC) assay

The TPC of the VAE was assayed using the Folin-Ciocalteau method, with some modifications (23). A 20 μL aliquot of the extract was added to 100 μL Folin-Ciocalteau reagents and 300 μL 20% Na_2CO_3 solution, and distilled water was added to a final volume of 2 mL. After 2 h, the absorbance was measured at 765 nm, and the concentration of TPC expressed as gallic acid equivalents (GAE) was determined using a calibration curve graphed following the same procedure, with gallic acid as a standard polyphenol.

Cell culture and treatment

Human basophilic KU812F cells were acquired from the American Type Culture Collection (Manassas, VA, USA), and maintained in RPMI-1640 medium supplemented with 10% FBS, HEPES (10 mM), penicillin (100 U/mL), and streptomycin (100 μg/mL), at 37°C in a humidified atmosphere with 5% CO_2, and passaged every 3～4 days. The cells were cultured in serum-free RPMI-1640 medium with or without various concentrations of the extract for 24 h.

Cell viability

Cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxylmethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay using the CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay in accordance with the manufacturer's instructions. KU812F cells were seeded on 96-well plates at a density of 2.5×10^4 cells/well, and incubated with serum-free medium in the presence of various concentrations of VAE for 24 h. The culture medium was removed and replaced with 95 μL of fresh culture medium and 5 μL of MTS solution. The cells were incubated for 1 h and the absorbance was measured at 490 nm using a microplate reader (VersaMax, Molecular Devices, Sunnyvale, CA, USA). Relative cell viability was calculated and compared with the absorbance seen with untreated cells. Each determination was made in triplicate and the data were expressed as the mean±standard deviation (SD).

Flow cytometry analysis

The cell surface expression of FcεRI was evaluated using flow cytometry. In brief, the pretreated KU812F cells (1×10^6) were harvested and incubated with 100 μL of anti-FcεRI α chain antibody, CRA-1 (10 μg/mL) on ice for 1 h. The cells were then washed with ice-cold phosphate-buffered saline (PBS) and stained with 100 μL of FITC-conjugated $F(ab')_2$ goat anti-mouse IgG (20 μg/mL), washed in ice-cold PBS, and subjected to flow cytometry (Epics® XL™, Beckman Coulter, Inc., Brea, CA, USA). A murine IgG antibody (10 μg/mL, Jackson ImmunoResearch Laboratories, Inc.) was used as a negative control. The percentage of FcεRI-positive cells was calculated with an arbitrary cutoff position of 2%, as determined by the negative control. The percentage of cells expressing FcεRI on the cell surface is shown in Fig. 1～5, which are representative of three independent experiments.

Reverse-transcriptase polymerase chain reaction (RT-PCR)

FcεRI α chain mRNA levels were determined using RT-PCR. KU812F cells (1×10^6) were treated with various concentrations of VAE for 24 h, were harvested and the pellet was then washed twice with PBS. Total cellular

RNA was isolated using the TRI$_{ZOL}$ reagent in accordance with the manufacturer's instructions. For cDNA synthesis, 1 μg of total RNA was added to RNase free water and 1 μL of 0.5 μg/μL of oligo (dT) primer, denatured at 70°C for 5 min, and cooled immediately. And then RNA was reversed transcribed in a master mix containing 4 μL of RT buffer, 1 μL of 10 mM dNTP and 1 μL of MMLV reverse transcriptase at 42°C for 50 min and at 70°C for 15 min. One μL of resultant cDNA samples were subjected to PCR amplification in the presence of specific sense and antisense primers. Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. The primer sequences used in this study are as follows: for the FcεRI α chain, sense 5'-CTT AGG ATG TGG GTT CAG AAG T-3' and antisense 5'-GAC AGT GGA GAA TAC AAA TGT CA-3'; for GAPDH, sense 5'- GCT CAG ACA CCA TGG GGA AGG T-3' and antisense 5'-GTG GTG CAG GAG GCA TTG CTG A-3'. The PCR reaction was conducted as follows; denaturation, 94°C for 30 s; annealing, 55°C for 30 s; and extension, 72°C for 1 min, and subjected to 18 cycles for the FcεRI α chain and GAPDH genes. The amplified PCR products were visualized via agarose gel electrophoresis and ethidium bromide staining, and subsequently analyzed using a Molecular Imager® Gel Doc™ XR System (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Western blot analysis

Expression of FcεRI protein and phosphorylation of mitogen-activated protein kinase (MAPK) were assessed by Western blot analysis. The treated and stimulated cells were washed with cold-PBS and lysed in 40 μL of cell lysis buffer containing 20 mM Tris-Cl (pH 8.0), 137 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM Na_3VO_4, 1 mM NaF, 2 mM ethylenediaminetetraacetic acid, and protease inhibitor cocktail. The protein samples were then subjected to 10% sodium dodecyl sulfate-polyacrylamide, and electrotransferred to a PVDF membrane. The membrane was immunoblotted using CRA-1, and anti-phosphorylated ERK 1/2, p38, or c-Jun N-terminal kinase (JNK) antibodies, followed by an HRP-conjugated anti-mouse IgG or an HRP-conjugated anti-rabbit secondary antibody. For detection, the chemoreactive proteins were visualized using enhanced chemiluminescence detection reagents in accordance with the manufacturer's instructions. The membranes were exposed to X-ray film and quantitated with a Molecular Imager® Gel Doc™ XR System.

Calcium influx assay

The intracellular Ca^{2+} concentration was measured using the calcium reactive fluorescence probe, fura 2-AM. KU812F cells (1×10^6) were treated with different concentrations of VAE suspended in 100 μL of Tyrode's buffer (137 mM NaCl, 2.7 mM KCl, 0.4 mM NaH_2PO_4, 1 mM $MgCl_2$, 12 mM $NaHCO_3$, and 1.8 mM $CaCl_2$) containing 2.0 μM fura 2-AM at 37°C for 30 min. The cells were then washed three times with PBS and stimulated with 100 μL of 10 μg/mL CRA-1. The fura 2 fluorescence was monitored with a microplate fluorescence reader (FLx800, BioTek Instruments, Inc., Winooski, VT, USA) at an excitation wavelength of 360 nm and an emission wavelength of 528 nm.

Histamine release assay

The histamine content was assessed using a spectrofluorometric assay (24). The pretreated cells (1×10^6) were suspended in Tyrode's buffer and stimulated with 100 μL of 10 μg/mL CRA-1 for 30 min at 37°C. Following centrifugation, the supernatant (100 μL) were collected and 40 μL of 1 N NaOH and 20 μL of 0.2% OPA were added. The mixtures were incubated on ice for 40 min and the reaction was terminated via the addition 10 μL of 3 N HCl. The fluorescence intensity was measured using a microplate fluorescence reader at an excitation wavelength of 360 nm and emission wavelength of 450 nm.

Statistical analysis

All measurements were conducted independently, in triplicate. The data are expressed as the mean±SD. The statistical differences between the control and VAE groups were determined via a Student's *t*-test, and were considered statistically significant at $P\leq0.01$.

RESULTS AND DISCUSSION

Effects of VAE on KU812F cell viability

V. angustifolium has been previously used for the treatment of diabetic symptoms, and it has been shown to exert a variety of physiological effects including antioxidant, anticancer, and anti-microbial effects (9-16). Methanol was found to be the most suitable solvent for the extraction of polyphenolic compounds from plant materials (25). The TPC of VAE was 170±1.9 mg GAE/g (data not shown). KU812F cells, a human basophilic cell line originally isolated from chronic myelocytic leukemia, expresses a high affinity IgE receptor, and thus it is recognized as a useful cell type for FcεRI expression studies (26).

To determine the precise activity of VAE, the viability of KU812F cells was assessed by the MTS assay using a CellTiter 96® AQ$_{ueous}$ One Solution Cell Proliferation Assay kit. VAE evidenced no cytotoxic effects in the concentration range of 1~20 μg/mL (Fig. 1).

VAE negatively regulates the FcεRI expression

FcεRI is a high-affinity IgE receptor expressed on the surface of mast cells and basophils as effector cells, and

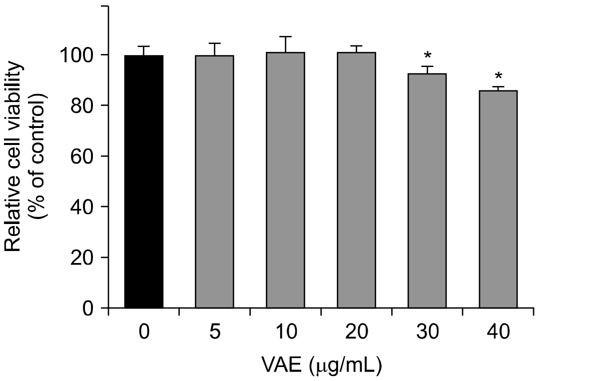

Fig. 1. Cytotoxic effect of *Vaccinium angustifolium* root extract (VAE) in KU812F cells. KU812F cells were cultured in the presence of different concentrations of VAE for 24 h under serum-free conditions, and the cell viabilities were determined via MTS assay. Each determination was made in triplicate and the data were expressed as the mean±SD. *Values are significantly different from control ($P<0.05$).

performs an important function in IgE-mediated allergic reactions (18-22). In order to assess the VAE-mediated suppression of FcεRI cell surface expression, KU812F cells were treated with different concentrations of VAE for 24 h under serum-free conditions, and the FcεRI cell surface expression was measured by flow cytometry using the anti-FcεRI α chain antibody, CRA-1. The FcεRI expression on the cell surface was reduced from 29.9% to 26.4%, 23.5%, and 17.9% upon treatment with VAE at 0, 5, 10, and 20 μg/mL, respectively (Fig. 2A). VAE was shown to suppress the cell surface expression of FcεRI in KU812F cells in a concentration-dependent manner.

Next, the inhibitory effect of VAE on FcεRI α chain expression in KU812F cells was confirmed by measuring the protein levels using Western blotting analysis (Fig. 2B). The decrease in FcεRI expression by VAE was found to be concentration-dependent. Additionally, the VAE-mediated suppression of FcεRI α chain gene expression was evaluated by measuring the mRNA levels of FcεRI α

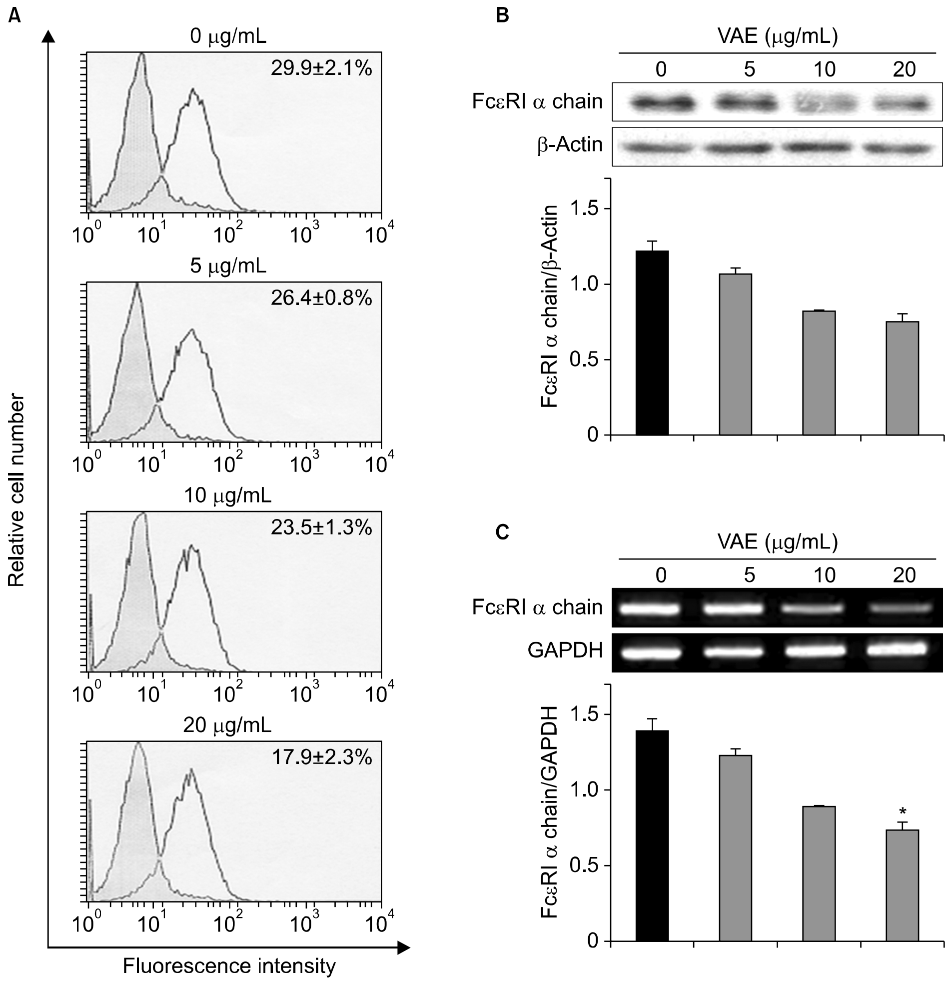

Fig. 2. Effects of *Vaccinium angustifolium* root extract (VAE) on expression of cell surface, protein, and mRNA level for the FcεRI α chain. KU812F cells were cultured in the presence of different concentrations of VAE (0, 5, 10, and 20 μg/mL) for 24 h. (A) Flow cytometry analysis was conducted using anti-FcεRI α-chain monoclonal antibody (CRA-1) followed by staining with FITC-conjugated $F(ab')_2$ goat anti-mouse immunoglobulins. (B) Western blot analysis was conducted using CRA-1 and β-actin. The amount of protein in each band was quantified by densitometry. (C) Total RNA was prepared, FcεRI α chain and GAPDH was detected by RT-PCR. Each value represents the mean±SD of three different experiments. *Values are significantly different from control ($P<0.05$).

chain by RT-PCR using the total cellular RNA. The FcεRI α chain mRNA in the untreated cells was clearly detected, and the corresponding mRNA levels of the VAE-treated cells were visibly reduced (Fig. 2C). The suppression of cell surface and total FcεRI protein expression in the presence of VAE was determined to be the consequence of a reduction in total cellular FcεRI α chain gene expression. The FcεRI α chain is expressed in FcεRI-positive cells and is known to be crucial for the proper functioning of the cell surface receptor for IgE (5). Gene expression of the FcεRI α chain, as determined by the mRNA level using RT-PCR, was shown to be downregulated by VAE. Gene expression of the FcεRI α chain is known to be regulated by at least two transcription factors, GATA and E74-like factor, in rodents and mammals, including humans (27,28). Further studies regarding the regulation of the transcription initiation signals of the gene encoding the FcεRI α chain are necessary in order to better understand the molecular regulatory mechanisms of VAE-mediated FcεRI expression.

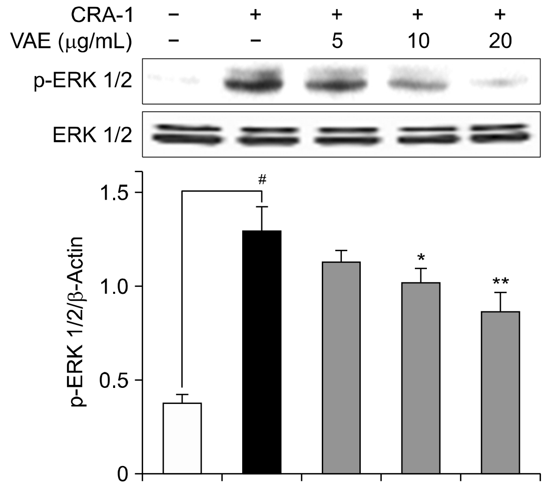

Fig. 3. Effects of *Vaccinium angustifolium* root extract (VAE) on phosphorylation of FcεRI-mediated ERK 1/2. Cells were treated with VAE, and stimulated with FcεRI α chain antibody (CRA-1). The cellular lysate was obtained, and the protein expression was assessed via Western blot analysis using anti-phospho-extracellular signal-regulated kinases (ERK) 1/2 and ERK 1/2 antibodies. Results are presented as the mean±SD of three independent experiments. Values are significantly different the control ($^{\#}P<0.05$) and CRA-1-treated control ($^{*}P<0.05$ and $^{**}P<0.01$).

Effects of VAE on FcεRI-mediated phosphorylation of ERK 1/2

The signaling pathway activated by the Fc receptors in mast cells and basophils has been extensively characterized. Initial FcεRI stimulation on the surface of these cells triggers a signaling cascade that includes activation of various transcription factors (29,30). In order to analyze the signaling pathways modulated by VAE, we also assessed the effects of VAE on the FcεRI-mediated phosphorylation of the MAPKs, ERK 1/2, p38 MAPK, and JNK. The cells were treated with VAE (0, 5, 10, and 20 μg/mL), stimulated with CRA-1, and the activities of the MAPKs were assessed by Western blotting analysis. In unstimulated KU812F cells, the levels of MAPK proteins were undetectable. VAE inhibited ERK 1/2 phosphorylation in a concentration-dependent manner (Fig. 3), however, it did not affect the phosphorylation of p38 MAPK or JNK (data not shown).

The MAPK cascade is one of the most important signaling pathways in immune responses (31). The suppression of cell surface FcεRI expression in the presence of VAE was attributed to a reduction in ERK 1/2 activation. Recently, the inhibition of MAPK phosphorylation by components such as (−)-epigallocatechin-3-*O*-gallate was shown, and cornuside has been associated with the expression of FcεRI (32,33). In the present study, we determined that the inhibition of FcεRI expression by VAE was associated with the down-regulation of ERK 1/2 phosphorylation. Considering the role of FcεRI in IgE-mediated allergic reactions, the suppression of allergen-IgE-FcεRI complex formation by VAE should be useful in the prevention of allergic diseases. In basophils and mast cells, the aggregation of FcεRI induces the activation of protein tyrosine kinases such as Syk, Lyn, phospholipase Cγ, and phosphatidylinositol-3 kinases. Therefore, further studies regarding the regulation of other signaling pathway factors by VAE is needed.

Effects of VAE on FcεRI-mediated [Ca^{2+}]*i* influx

Intracellular Ca^{2+} is important for the induction of mast cell and basophil degranulation that occurs via FcεRI crosslinking, leading to the phosphorylation of several protein tyrosine kinases and the activation of downstream signaling cascades, including Ca^{2+} influx (34). In order to determine the effects of VAE on intracellular Ca^{2+} influx, KU812F cells were labeled with the calcium-specific fluorescence probe, fura 2-AM, and stimulated with CRA-1. In VAE-treated cells, the intracellular Ca^{2+} concentration in the CRA-1-stimulated cells was reduced in a concentration-dependent manner (Fig. 4). FcεRI crosslinking activates downstream signaling cascades, including intracellular Ca^{2+} influx, which is required for degranulation (35). We demonstrated that VAE treatment concentration-dependently inhibited FcεRI-mediated intracellular Ca^{2+}. Moreover, FcεRI crosslinking activates protein tyrosine kinases including Syk and Lyn, and the phosphorylation of numerous proteins in mast cells and basophils (21,34,35). Therefore, in order to better understand the inhibitory mechanism of FcεRI expression via VAE, further studies regarding the regulation of transcription factors, such as protein tyrosine kinases, are needed.

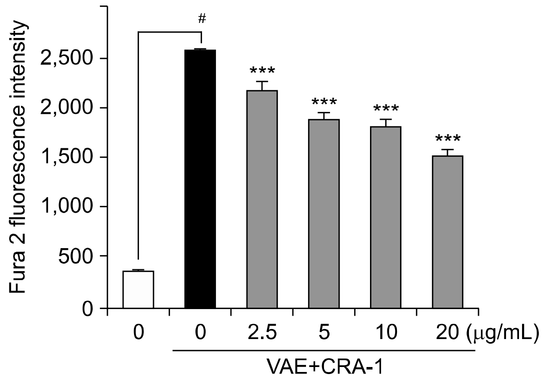

Fig. 4. Effects of *Vaccinium angustifolium* root extract (VAE) on FcεRI-mediated $[Ca^{2+}]i$ elevation. The pretreated cells with VAE were incubated with fura 2-AM and stimulated for 30 min with FcεRI α chain antibody (CRA-1), and $[Ca^{2+}]i$ was determined spectrofluorometrically. Each value is expressed as the mean± SD of three different experiments. Values are significantly different from control the control ($^{\#}P<0.05$) and CRA-1-treated control ($^{***}P<0.001$).

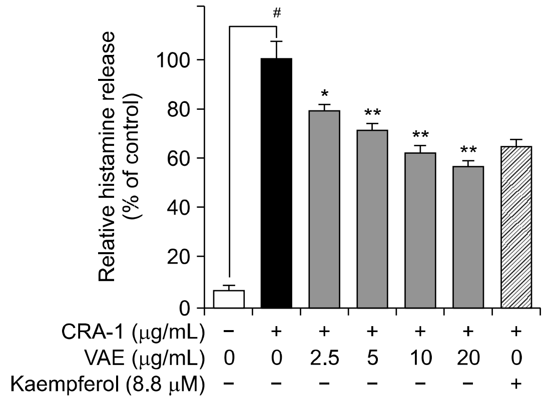

Fig. 5. Effects of *Vaccinium angustifolium* root extract (VAE) on FcεRI-mediated histamine release. The pretreated-cells with VAE were stimulated for 30 min with FcεRI α chain antibody (CRA-1) in Tyrode's buffer. Histamine content was determined via a spectrofluorometric method. Each value represents the mean±SD of three different experiments. Values are significantly different from the control ($^{\#}P<0.05$) and CRA-1-treated control ($^{*}P<0.05$ and $^{**}P<0.01$).

VAE inhibits FcεRI-mediated histamine release

The aggregation of FcεRI with an anti-FcεRI antibody, or multivalent allergen and IgE complexes, initiates a cascade of biochemical events that results in degranulation, inducing the secretion of inflammatory mediators such as histamine and β-hexosaminidase, and contributing to allergic responses (18-22). Histamine is an inflammatory mediator that is stored in secretory granules and released in immunologically-activated mast cells and basophils. Thus, histamine in the medium is utilized as a marker of the degranulation of mast cells and basophils (36). In order to assess the inhibitory effects of VAE on degranulation, together with kaempferol as a positive control, KU812F cells were treated with VAE, followed by stimulation with CRA-1, and the amount of histamine released from the cells was determined spectrofluorometrically using OPA. Treatment with VAE inhibited the histamine release from CRA-1-stimulated KU812F cells in a concentration-dependent manner (Fig. 5). The FcεRI-mediated degranulation of mast cells and basophils is closely related to FcεRI activation. VAE-induced modulation of FcεRI expression may be of particular importance in IgE-mediated allergic reactions because such effects elucidated in the present study can theoretically influence all FcεRI-mediated downstream signaling events. Here we demonstrated that the down-regulation of FcεRI expression by VAE leads to the inhibition of FcεRI-stimulated Ca^{2+} influx and histamine release, and these inhibitory effects may be exerted through the suppression of downstream signaling events that occur following FcεRI activation.

The root of *V. angustifolium* contains a variety of phenolic compounds including procyanidins, catechin, vanillic acid, chrorogenic acid, and epicatechin (4). Therefore, we believe that in order to better understand the VAE-mediated down-regulation of FcεRI expression, further studies of its bioactive compounds must be conducted.

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AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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